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Downregulation of Bcr-Abl in K562 cells restores susceptibility to apoptosis: Characterization of the apoptotic death

Anne J. McGahon[1], David G. Brown[2], Seamus J. Martin[1], Gustavo P. Amarante-Mendes[1], Thomas G. Cotter[3], Gerald M. Cohen[2] and Douglas R. Green[1,4]

[1] Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 11149 N. Torrey Pines Rd., La Jolla, CA 92014, USA
[2] The MRC Toxicology Unit, Center for Mechanisms of Human Toxicity, University of Leicester, Leicester, UK
[3] Tumor Biology laboratory, Biochemistry Department, University College Cork, Ireland
[4] corresponding author: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA, 92037, USA. tel: (619) 558-3500, fax: (619) 558-3525

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Abstract

We examined the susceptibility of a variety of human leukemic cell lines to the induction of apoptosis. K562, a chronic myelogenous leukemic cell line which expresses the *bcr-abl* fusion gene, was found to be extremely resistant to apoptosis, irrespective of the inducing agent. This resistance can be attributed to the deregulated Abl kinase activity of *bcr-abl*, as downregulation of its expression using antisense oligodeoxynucleotides targeted to the beginning of the *abl* sequence in this chimeric gene rendered these cells susceptible to cytotoxic drug-induced apoptosis. Examination of the morphological and biochemical features of apoptosis in K562 cells revealed the typical membrane blebbing and chromatin condensation associated with this form of cell death. *In situ* TdT-mediated end labeling of the DNA revealed the presence of strand breaks in the treated cells and field inversion gel electrophoresis revealed the presence of large 10–50 kb fragments. However there was an absence of oligonucleosomal DNA fragmentation, whether or not Bcr-Abl was expressed. Thus, while inhibition of expression of Bcr-Abl renders K562 cells susceptible to apoptosis, the absence of oligonucleosomal DNA fragmentation in these cells is independent of the function of this molecule.

Keywords: Bcr-Abl; apoptosis; DNA fragmentation; antisense

Abbreviations: CML, chronic myelogenous leukemia; PI, Propidium Iodide; AS, antisense; NS, nonsense; FIGE, field inversion gel electrophoresis

Introduction

Apoptosis is an active form of cell death and an important regulator of such diverse developmental processes as embryogenesis, metamorphosis, immune development and hematopoiesis (Kerr *et al*, 1972; Lockshin *et al*, 1991; Cohen *et al*, 1992). Apoptosis is characterized morphologically by cellular features such as membrane blebbing, chromatin condensation and cell shrinkage (Wyllie *et al*, 1980; Cohen *et al*, 1992). The cleavage of the DNA of an apoptotic cell into oligonucleosomal fragments is considered to be an important biochemical feature of this process in some but not all cell types (see Compton, 1992; Bortner *et al*, 1995 for recent reviews). More recently, large molecular weight DNA fragments (50–300 kbp in size) have been detected in apoptotic cells (Brown *et al*, 1993; Oberhammer *et al*, 1993; Walker *et al*, 1993; Cohen *et al*, 1994). This type of DNA fragmentation may precede internucleosomal DNA fragmentation (Oberhammer *et al*, 1993; Cohen *et al*, 1994), though the former can occur in the absence of the latter (Tomei *et al*, 1993).

While the deregulated growth characteristics of cancer cells are most often attributed to genetic aberrations in genes controlling cell proliferation or differentiation, overexpression of genes that suppress apoptotic cell death can also lead to increased cell numbers without any alteration in cell proliferation rates. Chronic myelogenous leukemia (CML) is a striking example of such a myeloaccumulative disorder. A shortened chromosome 22, the Philadelphia chromosome is observed in 90–95% of the leukemic cells of CML patients (Rowley, 1972). The Philadelphia chromosome is the product of a reciprocal exchange between the long arms of chromosomes 9 and 22. This fusion event results in a hybrid gene in which the amino-terminal sequence of the *bcr* gene on chromosome 22 is fused to the second exon of the *c-abl* gene on chromosome 22 is fused to the second exon of the *c-abl* gene on chromosome 9 (Groeffen and Heisterkamp, 1987). The resulting Bcr-Abl protein product has upregulated tyrosine kinase activity compared with the normal c-Abl protein (Konopka and Witte, 1985). Expression of *bcr-abl* has been demonstrated to cause lymphoid malignancies *in vitro* (McLaughlin *et al*, 1987; Young and Witte, 1988) and *in vitro* (Hariharan *et al*, 1989; Heisterkamp *et al*, 1990).

CML is characterized by the excessive accumulation of relatively mature cells in the bone marrow and blood stream. CML myeloid progenitors display normal mitotic indices, normal responses to colony-stimulating factors and do not proliferate faster than their normal counterparts (Stryckmans *et al*, 1976; Dormer *et al*, 1980; Koeffler and Golde, 1981; Strife and Clarkson, 1988). Hematopoietic cell population size is also regulated by apoptosis, for example hemopoetic colony stimulating factors suppress apoptosis (Williams, 1991; Greenberg, 1992), and thus the myeloid expansion seen in CML may be due to enhanced cell survival rather than excessive proliferation. The elevated

Abl tyrosine kinase activity observed in K562 cells may act to suppress apoptosis of these cells (Bedi *et al*, 1994; McGahon *et al*, 1994).

In the present study we examined the susceptibility of a number of human leukemic cell lines to cytotoxic drug-induced apoptosis. To determine if Bcr-Abl expression was responsible for the resistance to apoptosis observed in K562 cells, we adopted an antisense approach. Down-regulation of Bcr-Abl expression using antisense oligodeoxynucleotides corresponding to the *abl* sequence in *bcr-abl* mRNA rendered these cells susceptible to cytotoxic drug-induced death. We then utilized this antisense strategy to characterize the apoptotic cell death observed in these K562 cells. While conventional agarose gel electrophoresis failed to detect internucleosomal DNA fragmentation in K562 cells, field inversion gel electrophoresis revealed the presence of large molecular weight fragments. Our observations demonstrate that *bcr-abl* is acting as a negative regulator of apoptosis and large molecular weight DNA fragmentation in K562 cells.

Results

Resistance of K562 cells to apoptosis induced by cytotoxic drugs

Previous studies by ourselves (McGahon *et al*, 1994) and others (Bedi *et al*, 1994) had demonstrated a role for Bcr-Abl in the maintenance of resistance to apoptosis seen in chronic myelogenous leukemia (CML) cells. To further characterize this resistance, we extended our observations to a comparative study on the susceptibility of a number of human leukemic cell lines to the induction of apoptosis by cytotoxic agents. Figure 1 shows a dose-response of the cytotoxic agents on the human leukemic cell lines used. These included HL-60, a promyelocytic human leukemia cell line, Daudi and Raji, both B lymphoblastoid cell lines, Molt-3 and Jurkat, both T lymphoblastoid lines and K562, a cell line derived from a CML patient. The susceptibility to cell death following an overnight incubation with the various cytotoxic agents was measured by propidium iodide (PI) uptake. Each of the cell lines varied in their susceptibility to cell death reflecting different abilities to cope with toxic insult. The CML cell line K562 was the most resistant to cell death irrespective of the inducing agent.

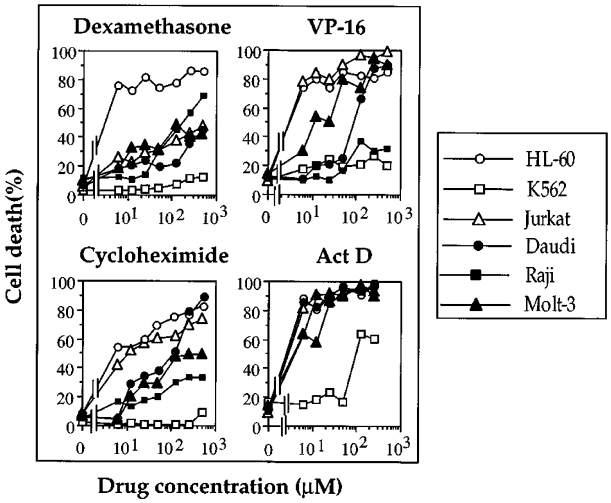


Figure 1 Dose dependent killing of human leukemic cell lines with cytotoxic drugs. Cells (1×10^5/ml) were seeded in 96 well plates and incubated overnight in varying concentrations of cytoxic agents. Cell viability was assessed by PI uptake as described in Materials and Methods.

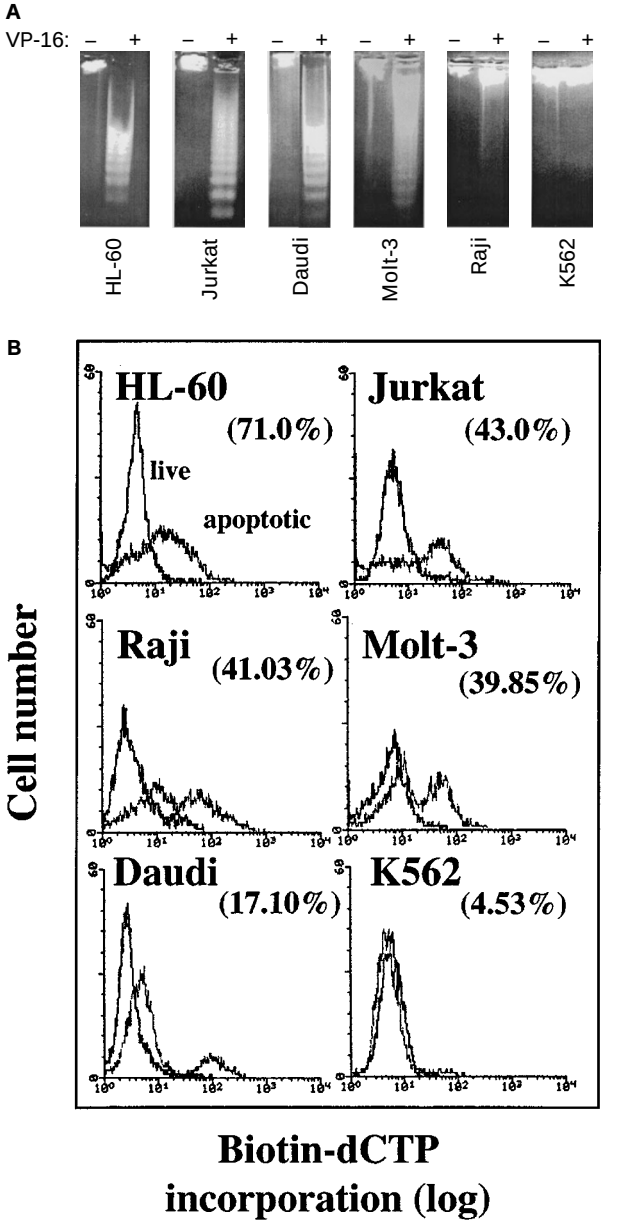


Figure 2 (A) Agarose gel electrophoresis of DNA extracted from untreated, and VP-16-treated cells. The human leukemic cell types indicated were subjected to an overnight incubation in the presence of 10 μM VP-16. DNA from 5×10^5 cells was electrophoresed through 1.5% agarose gels and stained with 10 ng/ml ethidium bromide. (B) TUNEL staining of human leukemic cells treated with 10 μM VP-16 for an 18 h period. Cells (1×10^6) were fixed and DNA strand breakage was analyzed by the ability of the cells to incorporate biotin-dCTP which was detected by fluorescinated avidin. Cell fluorescence was measured using the LYSYS 11 software on a FACScan Flow Cytometer (Becton Dickinson).

Whether a cell dies by apoptosis or necrosis upon injury has been shown to be dictated in part by the dosage of the insult the cell receives (Lennon *et al*, 1991). In order to determine if the cell death we observed was apoptotic in nature, we examined the morphological features of apoptosis in each cell type. The criteria for the identification of such apoptotic features included such features as membrane blebbing, chromatin condensation and formation of apoptotic bodies (Kerr *et al*, 1972; Wyllie *et al*, 1980). The dose response of the human cell lines to apoptotic cell death induced by the various cytotoxic agents was essentially identical to the effects seen by the criterion of PI uptake, above (results not shown). Again, K562 cells were consistently the most resistant to apoptosis induction among the cell lines examined.

A second characteristic feature of apoptosis in many but not all cells is the fragmentation of the DNA into oligonucleosomal fragments (for recent review, see Comp-

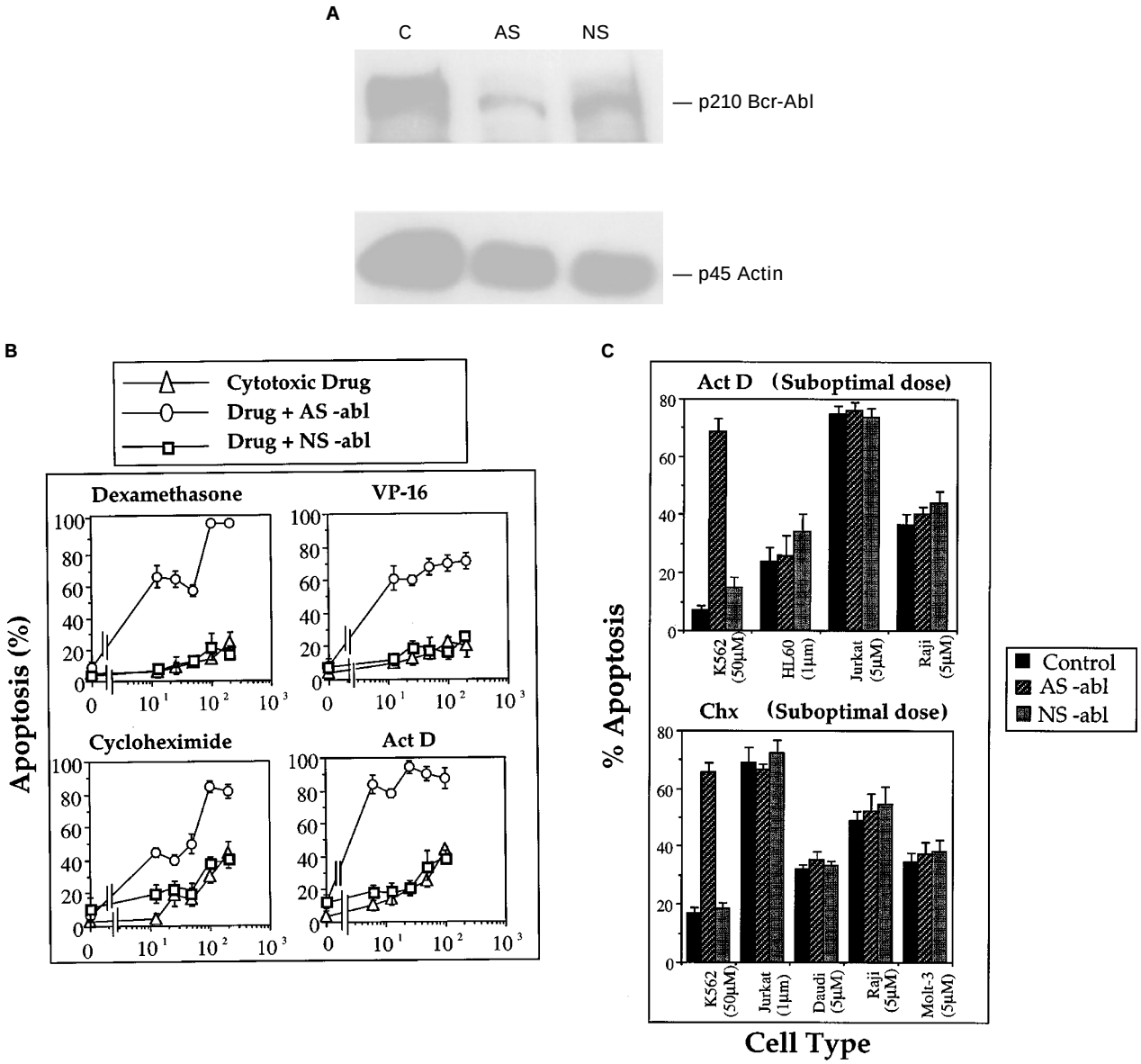
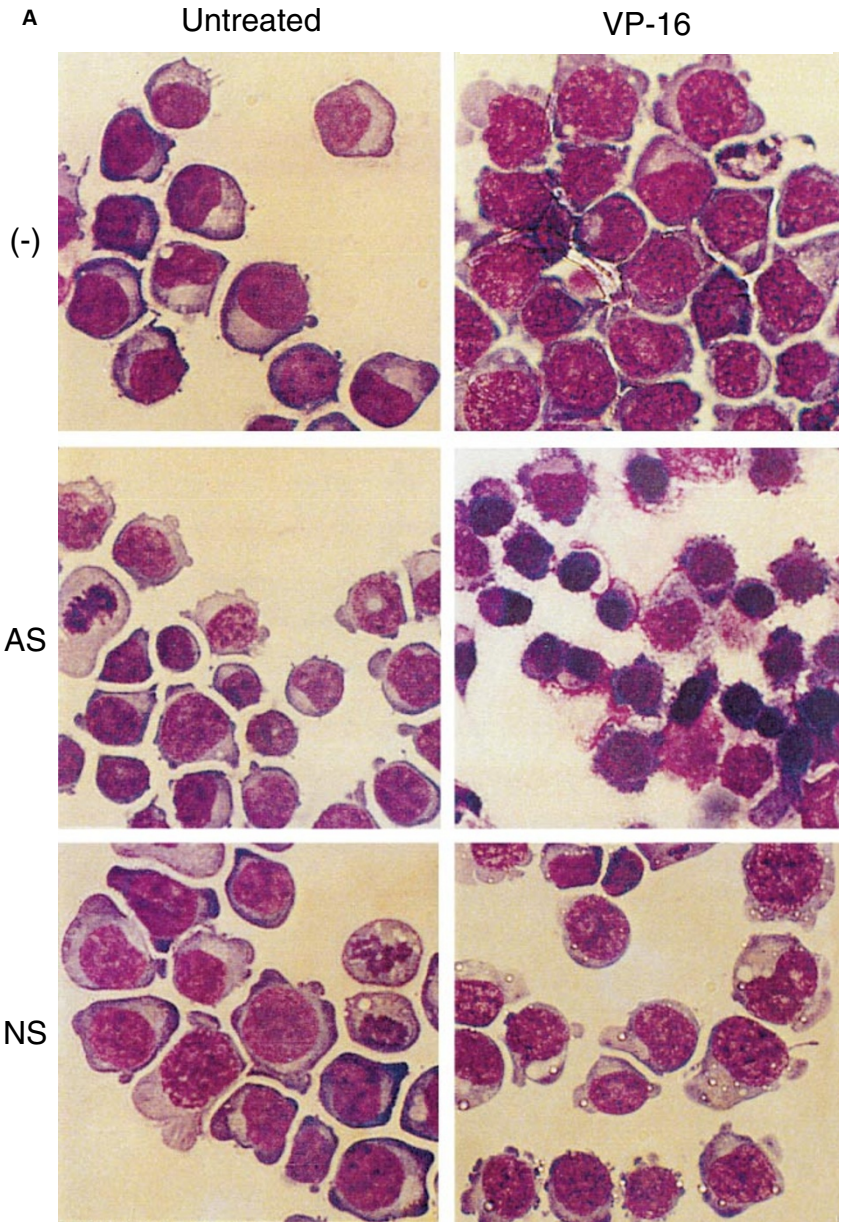


Figure 3 (A) Downregulation of Bcr-Abl protein levels in AS-abl treated K562 cells. K562 cells were either left untreated (Control) or incubated in the presence of 10 μM AS-abl (AS) or 10 μM NS-abl (NS) for 48 h. Cell lysates were prepared from the treated samples at the time points indicated and Bcr-Abl protein levels were examined by immunoblot analysis using anti-Abl (8E9) and HRP-conjugated anti-mouse IgG as described in Materials and Methods. The positions of Bcr-Abl protein p210 and Actin p45 are marked (B) Effect of AS-abl and NS-abl treatment on susceptibility of K562 cells to apoptosis induced by cytotoxic drugs. Untreated, AS-abl treated and NS-abl treated K562 cells (pretreated for 48 h) were incubated for 18 h in the presence of various concentrations of cytotoxic drugs and apoptosis was determined by morphological criteria as described in Materials and Methods. (C). Effect of AS-abl and NS-abl treatment on susceptibiility of human leukemic cell lines to apoptosis induced by sublethal doses of cytotoxic drugs. Untreated, AS-abl treated and NS-abl treated cells (pretreated for 48 h) were incubated for 18 h in the presence of sublethal concentrations of cytotoxic drugs and apoptosis was assessed as described in B.

ton, 1992). We therefore sought to determine if this biochemical feature was observable in the cell lines undergoing apoptosis. Agarose gel electrophoresis demonstrated the presence of the characteristic ladder pattern of DNA fragmentation in all the susceptible cell types except Raji (Figure 2A), these cells did not exhibit any DNA laddering pattern despite the morphological characteristics of apoptosis being present. Similar results were observed with all of the cytotoxic agents used (data not shown). Single-stranded DNA breakage has been observed in Raji cells upon CTL-mediated killing however (Gromkowski *et al*, 1986). This observation prompted us to examine the type of DNA damage occurring in our cell types upon the induction of apoptosis. In order to do so, we utilized an *in situ* Tdt-mediated DNA end labeling technique, TUNEL (Gorczyca *et al*, 1992). This technique demonstrated the presence of strand breaks in all of the cell types undergoing apoptosis, including Raji cells (Figure 2B). However, K562 cells failed to show any significant DNA cleavage as detected by this technique.

Downregulation of Bcr-Abl in K562 cells renders them susceptible to apoptotic cell death

We had previously demonstrated that antisense oligodeoxynucleotides corresponding to the translation start site of *bcr* could downregulate Bcr-Abl protein levels in K562 cells, and in turn render them susceptible to the induction of apoptosis (McGahon *et al*, 1994). While these studies indicated that the resistance to apoptosis observed in K562



cells was probably due to the activated Abl kinase activity, it remained a formal possibility that it could have been a property of the *bcr* gene alone. In order to examine this possibility, we therefore sought to determine if antisense oligodeoxynucleotides corresponding to the *abl* sequence (AS-abl) would have the same effect. As shown in Figure 3A, Bcr-Abl protein levels were found to be decreased in K562 cells after a pre-incubation of these cells with 10 μM AS-abl for a period of 48 h as previously reported (McGahon *et al*, 1994). In contrast, Bcr-Abl protein levels in K562 cells treated with 10 μM of a nonsense control (NS-abl) for the same time period remained comparable with Bcr-Abl levels in the untreated controls. Actin protein levels remained constant following treatment of both cell types with either the AS or NS oligodeoxynucleotides, confirming that the oligodeoxynucleotides used were specific for the Abl mRNA.

The effect of lowering Bcr-Abl protein levels in K562 cells on susceptibility to the induction of apoptosis was then examined. Untreated, AS-abl and NS-abl treated K562 cells were incubated overnight in varying concentrations of cytotoxic agents and apoptosis was assessed by examination of stained cytospun preparations of the treated cells (Figure 3B). K562 cells treated with AS-abl oligodeoxynucleotides underwent extensive cell death upon cytotoxic drug treatment (Figure 3B and C). In contrast, NS-abl treated or untreated cells remained resistant to apoptosis induced by any of these agents. AS or NS-abl treatment alone had no effect on cell viability.

It remained possible that the restoration of susceptibility to apoptosis seen in K562 cells was caused by a nonspecific effect of the antisense. To test this hypothesis, we pretreated all the human leukemic cell lines used in this study with 10 μM As-abl for a period of 48 h, prior to subjecting them to an overnight treatment with suboptimal doses of cytotoxic drugs. AS-abl treatment had no effect on susceptibility of these cell types to suboptimal doses of apoptosis-inducing agents (Figure 3C), indicating that the increased susceptibility seen only in the K562 cells was specific to effects on Bcr-Abl.

Absence of oligonucleosomal DNA fragmentation in K562 cells

Agarose gel electrophoresis of the DNA of antisense pretreated K562 cells subjected to cytotoxic agents revealed an absence of oligonucleosomal DNA fragmentation (results not shown), despite the presence of such characteristic morphological features as membrane blebbing and chromatin condensation (Figure 4A). Previous studies by our group had demonstrated a lack of internucleosomal DNA fragmentation in these cells (Fernandes and Cotter, 1993; McGahon *et al*, 1994). We did observe strand breaks in these cells however, as

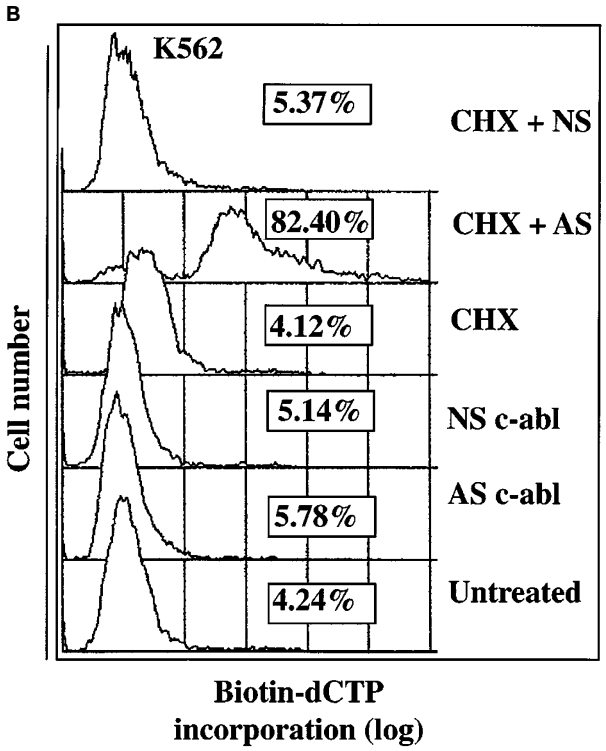


Figure 4 (A) Morphology of VP-16-mediated apoptosis in K562 cells viewed under light microscopy of stained cytospun preparations. Magnification 600×. Untreated, AS-abl treated and NS-abl treated cells (2.5×10^6/ml) were incubated for 18 h in the presence of 10 μM VP-16. Typical apoptotic features displayed by VP-16-treated cells include membrane blebbing and chromatin condensation. (B) TUNEL labelling of untreated, AS-abl treated and NS-abl treated K562 cells subjected to 10 μM Chx treatment for 18 h. Cells (1×10^6) were fixed and DNA strand breakage was analyzed as described in Figure 2B.

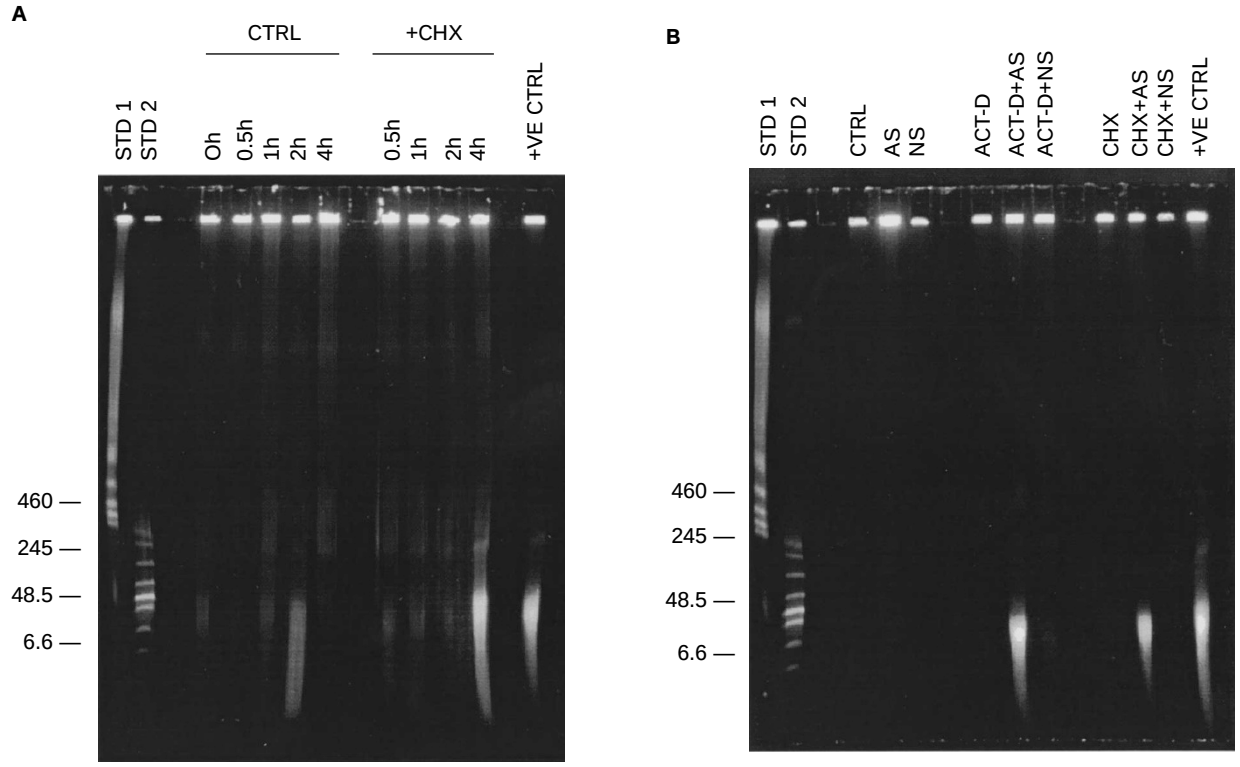


Figure 5 (A) Time course of high molecular weight DNA fragmentation in Chx-treated HL-60 cells as assessed by FIGE. Agarose plugs were prepared from 10^6 cells per treatment and electrophoresed under the conditions described in Materials and Methods. *Sacharomyces cerevisiae* chromosomes, 243-2200 kilobase pairs (Clontech, Cambridge, United Kingdom) and Pulse Marker 0.1 to 200 kilobase pairs (Sigma Chemical Co, Poole, United Kingdom) were used as standards. Rat thymocytes treated with 10 μM VP-16 were used as a positive control. (B) FIGE of DNA extracted from untreated, AS-abl treated and NS-abl treated K562 cells incubated in the presence of 10μM Act-D or 10 μM Chx to induce apoptosis. The standards used are described in (A) and again, rat thymocytes treated with 10 μM VP-16 were used as a positive control.

monitored by TUNEL (Figure 4B). These observations led us to further characterize the DNA fragmentation in K562 cells and to determine whether it was under the regulation of Bcr – Abl.

Regulation of high molecular weight DNA fragmentation in K562 cells by Bcr-Abl

A number of recent observations have demonstrated the absence of internucleosomal cleavage in some cell types undergoing apoptosis (Gromkowski *et al*, 1986; Oberhammer *et al*, 1993; Tomei *et al*, 1993). Field inversion gel electrophoresis (FIGE) has identified the presence of high molecular weight DNA fragments in some of these cell types (Walker *et al*, 1991, 1993; Brown *et al*, 1993; Cohen *et al*, 1994). We employed this FIGE technique to determine whether K562 cells were undergoing high molecular weight DNA fragmentation, following the induction of apoptosis. HL-60 cells were used as a positive control in our experiments as these cells had previously been demonstrated to fragment their DNA into large molecular weight fragments prior to undergoing oligonucleosomal DNA fragmentation (Brown *et al*, 1993). Figure 5A shows a time course of the appearance of high molecular weight DNA fragments in HL-60 cells treated with 10 μM Chx. FIGE of antisense treated K562 cells subjected to VP-16 and Chx treatment (10 μM) revealed the presence of high molecular weight DNA fragments of approx. 50 kbp in these cells (Figure 5B). Untreated or NS-abl treated K562 cells subjected to similar treatment did not show this pattern of DNA fragmentation. Taken together our observations suggest that the morphological appearance of apoptosis, loss of cell viability, and appearance of strand breaks detected by TUNEL are all associated with high (but not low) molecular weight DNA fragmentation in K562 cells. Furthermore, these results demonstrate a regulation of apoptosis and high molecular weight DNA fragmentation in K562 by Bcr-Abl.

Discussion

In the present study we examined the relative susceptibilities of various human leukemic cell lines to the induction of apoptosis by cytotoxic drugs. K562 cells were highly resistant to apoptosis irrespective of the inducing agent. Using antisense oligodeoxynucleotides targeted to the *abl* region of *bcr-abl*, we have shown that downregulation of Bcr-Abl protein levels in K562 cells renders these cells highly susceptible to apoptosis induced by a variety of cytotoxic drugs. Furthermore, the increased susceptibility to apoptosis upon Bcr-Abl downregulation is a specific effect of the anitsense itself, as AS-abl treatment had no effect on the susceptibilities of a number of *bcr-abl*-negative cell types to the induction of apoptosis. The present observations

represent a formal demonstration that *bcr-abl* acts as a broad spectrum anti-apoptotic gene in K562 cells.

Carlesso and colleagues (1994) demonstrated that transfection of 32Dc13 cells with a temperature sensitive kinase mutant of *bcr-abl* protects against cell death induced by growth factor withdrawal at the permissive temperature for the kinase only. Similarly, transfection of an IL-3 dependent cell line (IC.DP) with a temperature sensitive v-Abl mutant renders these cells resistant to apoptosis following IL-3 withdrawal, only at the permissive temperature for the v-Abl kinase (Evans *et al*, 1993). Recent studies by ourselves* and others (Evans *et al*, 1993; Chapman *et al*, 1994) have demonstrated that the v-Abl tyrosine kinase can suppress apoptosis in a number of cell types. Thus, v-Abl has been shown to protect against apoptosis in IC.DP cells (Chapman *et al*, 1994), the human leukemic HL-60 cell line and the A1.1 T cell hybridoma (Green *et al*, 1994; McGahon *et al*, submitted*). Recently, Evans and colleagues (1995) suggested that the ability of v-Abl to block apoptosis is dependent upon an associated activation and nuclear translocation of protein kinase C, although whether this is a general requirement remains unresolved.

Antisense oligonucleotides which downregulate expression of Bcr-Abl have been shown to render K562 cells susceptible to several forms of apoptosis (McGahon *et al*, 1994), and another cell line, Bv173, spontaneously undergoes apoptosis upon treatment (Smetsers *et al*, 1994). It is possible that culture conditions of the latter cells provide a stress that induces apoptosis which is blocked by Bcr-Abl, such that downregulation of this molecule now allows the cells to die. Similarly, Bedi and colleagues (1994) showed that hematopoietic progenitors from CML patients are relatively resistant to induction of apoptosis, and that antisense treatment can sensitize these cells. This resistance to apoptosis of hematopoietic progenitors from CML patients is, however, controversial (Amos *et al*, 1995). More recently, Bedi and colleagues (1995) have suggested that the anti-apoptotic effect of Bcr-Abl is related to a delayed progression through G2/M, allowing time for DNA repair and prevention of mitotic catastrophe. While this may help to explain resistance to apoptosis induced by DNA-damaging agents, it is unlikely to explain resistance to other forms of apoptosis, such as induced by glucocorticoids (McGahon *et al*, 1994) or ligation of Fas/CD95 (McGahon *et al*, 1995b).

Degradation of the DNA into oligonucleosomal size fragments (180–200 bp in length) has served as a biochemical hallmark of apoptosis in many cells (Wyllie and Morris, 1982). In our present study we observed chracteristic morphological features of apoptosis in all of the human leukemic cell lines examined. These included cell shrinkage, membrane blebbing and chromatin condensation (Wyllie *et al*, 1980), however these cell lines varied in their ability to fragment their DNA into oligonucleosomal size fragments. Agarose gel electrophoresis of the DNA of the treated cells failed to reveal a ladder pattern in Raji cells (Figure 2A), despite the morphological features of apoptosis being present (data not shown). A number of observations demonstrate the occurrence in some model systems of the morphological appearance of apoptosis in the absence of a detectable DNA ladder (Wyllie and Morris, 1982; Oberhammer *et al*, 1993; Zakeri *et al*, 1993). Single-stranded DNA breakage has been observed in some cell types including Raji cells, upon CTL-mediated killing (Gromkowski *et al*, 1986). Using an *in situ* DNA end labeling technique we demonstrated the presence of DNA strand breaks in Raji cells undergoing apoptosis in response to VP-16 treatment (Figure 2B). Our observations demonstrate the need for the use of both morphological and biochemical assays in the study of apoptosis.

Several research groups have detected large DNA fragments of 50–300 kb during apoptosis (Walker *et al*, 1991; Brown *et al*, 1993; Oberhammer *et al*, 1993; Cohen *et al*, 1994). It has also been demonstrated that large molecular weight DNA fragmentation occurs before internucleosomal DNA cleavage and that these large DNA fragments serve as precursors for the smaller DNA fragments (Walker *et al*, 1991; Brown *et al*, 1993; Oberhammer *et al*, 1993; Cohen *et al*, 1994). We failed to see oligonucleosomal DNA fragmentation in K562 cells upon rendering them susceptible to cytotoxic drug-induced apoptosis (results not shown) despite the presence of the morphological features of apoptosis (Figure 4). Using FIGE we detected large molecular weight DNA fragments in apoptotic K562 cells. Our observations lead us to conclude that the induction of apoptosis and the accompanying large molecular weight DNA fragmentation observed in K562 cells is under the regulation of *bcr-abl*. Furthermore since *bcr-abl* can suppress the large molecular weight DNA fragmentation seen in K562 cells, our results strongly suggest that it is acting upstream of such early DNA degradative events.

Kinase deregulation in v-Abl and Bcr-Abl results in oncogenic conversion (Suen *et al*, 1990) suggesting that the regulation of c-Abl tyrosine kinase is critical to the maintenance of a normal cell phenotype. These studies would argue that the basic function of c-Abl remains unchanged and that the generation of a hybrid *bcr-abl* gene results in an exaggeration of normal Abl function. The present study demonstrates that the resistance to apoptosis seen in CML cell lines is due to the expression of Bcr-Abl. Therefore it is possible that one normal cellular function of Abl is as a negative regulator of apoptosis. Support for this hypothesis comes from the observations that a homozygous null mutation of c-Abl results in neonatal mortality and a loss of lymphocyte longevity (Schwartzberg *et al*, 1991), suggesting that c-Abl may function as a negative regulator of apoptosis in lymphocytes. Deregulation of this function in myeloid cells results in steady state accumulation of cells that would normally undergo apoptosis (Bedi *et al*, 1994; McGahon *et al*, 1994). Further examination of the

*McGahon AJ, Amarante-Mendes GP, Martin SJ, Brunner T, Yoo NJ, Bissonette RP, Harigai M, Cotter TG, Reed JC and Green DR (1995) The v-abl kinase induces a novel anti-apoptotic state dependent upon an associated downregulation of Bax. Submitted.

normal cellular function of c-Abl and how it is regulated would provide insight into how it functions to control apoptosis and oncogenesis upon deregulation.

Materials and Methods

Cell culture and reagents

All cell lines described were grown in RPMI 1640 supplemented with 5% FCS and 2 mM L-glutamine. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere and were routinely subcultured every 2–3 days. All cytotoxic drugs used in this study were purchased from Sigma Chemical Co, St. Louis, MO. Stock solutions of both Actinomycin-D and VP-16 were prepared in RPMI media. Cycloheximide stock solutions were prepared as a 100 × stock in ethanol and dexamethasone stock solutions were prepared in ddH_2O. For the induction of apoptosis, cells (2.5×10^5/ml) were seeded in 96 well plates and incubated overnight in varying concentrations of the cytotoxic drugs.

Assessment of cell death and apoptosis

Cell death was quantitate by morphological assessment of stained cytospun preparations and also by flow cytometry. Leukostat (Fisher Scientific, Orangeburg, NY) stained cytocentrifuge cell preparations were examined for the morphological characteristics of apoptosis using previously defined criteria (Wyllie *et al*, 1980; McGahon *et al*, 1995a). Typical identifiable apoptotic features included membrane blebbing and chromatin condensation. Apoptosis was quantitated by scoring triplicate fields of cytospun samples.

The criteria for cell death as measured by flow cytometry was based on two parameters: changes in light scattering properties of dead cells due to cell shrinkage and increased granularity (Cotter *et al*, 1992) and their permeability to the DNA binding dye propidium iodide (PI) (Martin *et al*, 1994; McGahon *et al*, 1995a). Cells (2.5×10^5/ml) were incubated with 5 μl/ml PI at room temperature and analyzed directly on a Becton-Dickinson FACscan using LYSYS II software.

Antisense treatment of cell lines

Cells (2.5×10^5/ml) were incubated in the presence of either 10 μM AS-abl or 10 μM NS-abl for a 48 h time period, these conditions having been established previously as optimal for Bcr-Abl protein depletion in K562 cells (McGahon *et al*, 1994). Phosphorotioate derivatised oligodeoxynucleotides (QCB, USA) corresponding to the beginning of the *abl* sequence in *bcr-abl* were used. A random sequence of the same base composition as that of the AS oligonucleotide was used as a negative control. Oligonucleotide sequences used were as follows: AS-abl: 5′-TACTGGCCGCTGAAGGGC-3′; NS-abl: 5′-AGCATGTCGGACGTGG-3′.

Immunoblot analysis

For immunoblotting, 30 μg of total cellular lysate that had been resolved by SDS–PAGE was transferred onto an Immobilon filter as described previously (Martin *et al*, 1995). The filter was preblocked in TBS-Tween (Tris-buffered saline, 0.1% Tween-20) for 1 h at room temperature. Bcr-Abl protein levels were detected by probing with an anti-Abl antibody c-Abl Ab-3 (Oncogene Science, USA) at a 1:500 dilution for 1 h at room temperature. Following a 1 h wash in TBS-Tween, detection of the bound primary antibody was performed using an enhanced chemiluminscent blotting detection system (Amersham, USA). The filter was probed with a secondary antibody conjugated to horse radish peroxidase at 1:2000 dilution for 1 h, washed for 1 h and probed with ECL reagents before exposure to hyperfilm.

Agarose gel electrophoresis of nucleosomal DNA fragmentation

A total of 5×10^5 apoptotic cells or untreated control cells were washed, resuspended in 20 μl of lysis buffer (100 mM Tris (pH 8.0), 20 mM EDTA, 0.8% (w/v) sodium lauryl sarcosinate) plus 10 μl of RNase A (10 mg/ml), and incubated at 37°C for 30 min. Ten μl of Proteinase K (20 mg/ml) was then added and the cells were incubated for 2 h at 50°C. The resulting DNA was subjected to agarose gel electrophoresis in 1.5% agarose in TAE as described previously (Shi *et al*, 1992; McGahon *et al*, 1995a).

Field inversion gel electrophoresis (FIGE)

FIGE was used to resolve large molecular weight fragments of DNA. Agarose plugs containing 1×10^6 cells were prepared using standard techniques (Cohen *et al*, 1992, 1994; Oberhammer *et al*, 1993). The plugs were digested with Pronase (Boehringer Mannheim USA) (1 mg/ml) for 48 h at 50°C and were subsequently stored at 4°C until examined by FIGE as previously described (Brown *et al*, 1993). Under the conditions used, DNA fragments ranging in size from 4.4 to 460 kilobase pairs were resolved. *Saccharomyces cerevisiae* chromosomes, 243–2200 kilobase pairs (Clontech, Cambridge, United Kingdom) and Pulse Marker 0.1 to 200 kilobase pairs (Sigma Chemical Co. Poole, United Kingdom) were used as standards.

TUNEL staining

The DNA in individual cells was labelled with exogenous terminal deoxynucleotidyl transferase (TdT) using a modification of described methods (Gorczyca *et al*, 1992; McGahon *et al*, 1995a). Briefly, cells (5×10^5 ml) were fixed in 2% paraformaldehyde in PBS for 15 min at 4°C. The cells were then pelleted, washed in PBS and resuspended in 300 μl of PBS, followed by 700 μl of ice cold 100% ethanol. The cells were stored in the 70% ethanol solution at −20°C overnight before being subjected to the Tdt. After rehydration in PBS, the cell pellets were resuspended in 50 μl of the staining solution (0.1 mol/L sodium cacolydate (pH 7,0), 0.1 mol/L dithiothreitol, 0.05 mg/ml bovine serum albumin, 5 U of TdT and 0.5 nmol/L biotin dCTP), and incubated for 1 h at 37°C. After a PBS wash, the cells were resuspended in 100 μl of staining buffer (saline-sodium citrate buffer containing 0.1% Triton-X-100, 5% w/v) non fat dry milk and avidin-FITC, 2.5 mg/ml) and incubated at room temperature in the dark, for a further 30 min. Cell fluorescence was measured using the LYSYS II software on a FACscan Flow Cytometer (Becton Dickinson, San Jose, CA).

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